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γ-Adaptin Interacts Directly with Rabaptin-5 through Its Ear Domain[1]

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In yeast two-hybrid screening using γ1-adaptin, a subunit of the AP-1 adaptor complex of clathrin-coated vesicles derived from the *trans*-Golgi network (TGN), as bait, we found that it could interact with Rabaptin-5, an effector of Rab5 and Rab4 that regulates membrane docking with endosomes. Further two-hybrid analysis revealed that the interaction occurs between the ear domain of γ1-adaptin and the COOH-terminal coiled-coil region of Rabaptin-5. Pull down assay with a fusion protein between glutathione *S*-transferase and the ear domain of γ1-adaptin and coimmunoprecipitation analysis revealed that the interaction occurs *in vitro* and *in vivo*. Immunocytochemical analysis showed that γ1-adaptin and Rabaptin-5 colocalize to a significant extent on perinuclear structures, probably on recycling endosomes, and are redistributed into the cytoplasm upon treatment with brefeldin A. These results suggest that the γ1-adaptin–Rabaptin-5 interaction may play a role in membrane trafficking between the TGN and endosomes.

Key words: γ-adaptin, Rabaptin-5, recycling endosome, *trans*-Golgi network, yeast two-hybrid system.

In the exocytic and endocytic pathways of eukaryotic cells, membrane-bound compartments communicate by vesicular transport. Carrier vesicles that capture cargo molecules bud from a donor compartment and deliver their contents by fusing with an acceptor compartment. The vesicle budding requires the assembly of specific coat protein complexes onto the donor membrane. Three classes of coated vesicles have been well characterized to date: COP II–, COP I–, and clathrin-coated vesicles (for review, see Refs. *1–3*). The major constituents of clathrin-coated vesicles are clathrin and heterotetrameric adaptor protein (AP) complexes. Four AP or AP-related complexes have been identified so far, called AP-1, AP-2, AP-3, and AP-4 (for review, see Refs. *4–7*). The AP-1 complex is found at the *trans*-Golgi network (TGN) and is responsible for the delivery of lysosomal proteins, although there is evidence that AP-1 is also associated with endosomes (*8–11*) and with immature secretory granules in neuroendocrine cells (*12, 13*). The AP-2 complex is found at the plasma membrane and is involved in the endocytosis of cell surface receptors. The AP-3 complex appears to be involved in the delivery of some proteins to lysosomes and related compartments. The AP-4 complex is unable to associate with clathrin and its function is poorly understood.

Each AP complex is composed of two large subunits (more than 100 kDa), often called adaptins, one medium subunit (approximately 50 kDa), and one small subunit (approximately 20 kDa): γ- and β1-adaptins, μ1 and σ1 in AP-1; α- and β2-adaptins, μ2 and σ2 in AP-2; δ- and β3-adaptins, μ3 and σ3 in AP-3; and ε- and β4-adaptins, μ4 and σ4 in AP-4 (*4–7*). In addition, we and others recently identified a γ-adaptin homolog, designated γ2-adaptin (the original γ-adaptin was renamed γ1-adaptin as a consequence) (*14, 15*), which may constitute an AP-1 subtype in place of γ1-adaptin. On the basis of electron microscopic visualization of the AP-2 complex (*16*), adaptin polypeptides can be divided into two major domains, the NH_2-terminal head or trunk domain and the COOH-terminal ear or appendage domain, which are connected by a proline-rich hinge region. Each AP subunit is thought to play specific roles. The μ subunits are responsible for the recognition of tyrosine-based sorting signals within the cytoplasmic domains of transmembrane cargo proteins (*17, 18*; reviewed in Refs. *7* and *19*). β-Adaptins and γ-adaptins are able to interact with clathrin heavy chains through the clathrin-binding motifs within their hinge regions and promote clathrin assembly (*20–24*). α-Adaptin, through its ear domain, is capable of interacting with a wide variety of cytosolic molecules that regulate endocytic processes by the recognition of Asp-Pro-Phe/Trp motifs (*19*). γ1-Adaptin has recently been shown to interact with γ-synergin, the function of which is currently unknown (*25*).

On the other hand, the molecular machineries that regu-

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Abbreviations: AP, adaptor protein; ARF, ADP-ribosylation factor; BFA, brefeldin A; EEA1, early endosomal autoantigen 1; GST, glutathione *S*-transferase; HA, hemagglutinin; NRK, normal rat kidney; NSF, *N*-ethylmaleimide–sensitive factor; PBS, phosphate-buffered saline; SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor; TGN, *trans*-Golgi network.

late the docking and fusion of membranes have been elucidated in the past few years. NSF (*N*-ethylmaleimide–sensitive factor), SNAREs (soluble NSF attachment protein receptors), and the Rab family of small GTPases have been assigned special roles in these processes (for review, see Refs. *26* and *27*). The Rab family comprises more than 30 members in mammalian cells, and these have functions in different trafficking steps in the exocytic and endocytic pathways (reviewed in Refs. *28–30*). Among them, the most extensively studied is Rab5. Rab5 has been shown to regulate the docking and fusion of early endosomes by interacting with various effector molecules, including Rabaptin-5, early endosomal autoantigen 1 (EEA1), and phosphoinositide 3-kinases (*31–34*, reviewed in Ref. *30*). Recent evidence indicates that an oligomeric complex containing EEA1, Rabaptin-5, Rabex-5, and NSF drives membrane fusion *via* interactions between EEA1 and SNAREs, syntaxin 13 and syntaxin 6 (*35–37*). Although Rabaptin-5 was originally identified by its interaction with Rab5 (*31*), Rabaptin-5 and its variant, Rabaptin-4, were then shown to interact with Rab4 as well (*38, 39*). Rab5 and Rab4 interact with the COOH- and NH_2-terminal coiled-coil regions, respectively, of Rabaptin-5 (*31, 38, 39*), associate with distinct but partially overlapping endosomal structures, and may regulate distinct fusion processes (*40–42*).

In yeast two-hybrid screening with γ1-adaptin as bait, we found γ1-adaptin was able to interact with Rabaptin-5. In the present study, we therefore set out to characterize this interaction.

MATERIALS AND METHODS

Plasmid Construction—For use in yeast two-hybrid analyses, a bait vector for human γ1-adaptin (pGBT-γ1) was constructed as described previously (*14*). Bait vectors for deletion mutants of human γ1-adaptin (γ1-head, amino acid residues 1–594; and γ1-ear, amino acid residues 706–822), γ2-adaptin (γ2-head, amino acid residues 1–592; and γ2-ear, amino acid residues 669–785), and mouse αA-adaptin (αA-ear, amino acid residues 739–977) were constructed by separate subcloning of corresponding cDNA fragments obtained by a polymerase chain reaction-based method into pGBT9-BEN (*14*). Prey vectors for full-length mouse Rabaptin-5 and its deletion mutants (amino acid residues 276–546, 546–862, 546–728, and 662–862) were constructed by separate subcloning of corresponding cDNA fragments obtained by a polymerase chain reaction-based method into pGAD10 (CLONTECH Laboratories, Palo Alto, CA). To prepare GST-fusion proteins for use in pull down assays, cDNA fragments for γ1-ear, γ2-ear, and αA-ear were subcloned separately into pGEX-4T-2 (Amersham Pharmacia Biotech, Buckinghamshire, UK). Because the fusion protein between GST and the αA-ear (amino acid residues 739–977), which was used in the two-hybrid analysis, was insoluble in *Escherichia coli* cells, another fusion construct between GST and an αA-adaptin region covering a portion of the hinge region and the entire ear domain (amino acid residues 653–977) was used. For expression in mammalian cells as HA-tagged proteins, full-length mouse Rabaptin-5, human Rab4(Q67L), and human Rab5(Q79L) cDNAs were subcloned separately into pcDNA3-HAN (*43*) [the resulting plasmids were referred to as pcDNA3-HA-Rbt5, pcDNA3-HA-Rab4(Q67L), and pcDNA3-HA-Rab5-(Q79L), respectively].

Yeast Two-Hybrid Analysis—pGBT-γ1 was transformed by a lithium acetate–based method into yeast Y190 cells, which were plated for selection on synthetic medium lacking tryptophan. The transformant was then transformed with a mouse brain MATCHMAKER cDNA library (CLONTECH Laboratories), grown for 8 h in synthetic medium lacking tryptophan, leucine, and histidine, and plated on synthetic medium lacking tryptophan, leucine, and histidine and containing 25 mM 3-aminotriazole (Wako Pure Chemical Industries, Osaka). After 10 days of incubation, colonies were examined for β-galactosidase activity by a replica filter assay. Library plasmids from positive clones were rescued into *E. coli* HB101 cells by plating on leucine-free minimum medium, and subsequently analyzed by retransformation tests and DNA sequencing. By screening approximately 1×10^6 transformants, one positive clone was found to code for mouse Rabaptin-5 (amino acid residues 276–862). β-Galactosidase activity was also measured by a liquid culture assay using *o*-nitrophenylgalactopyranoside as a substrate (*44*). Two-hybrid analysis was also performed with the head or ear domain of γ1- or γ2-adaptin or the ear domain of αA-adaptin as bait, and one of the deletion mutants of Rabaptin-5 as prey.

Pulldown Assay—GST-fusion proteins of γ1-ear, γ2-ear, and αA-ear were expressed in *E. coli* BL21(DE3) cells and purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech). HeLa cells grown to approximately 80% confluence in nine 100-mm dishes were washed twice with ice-cold phosphate-buffered saline (PBS) and homogenized in 1 ml of homogenization buffer (20 mM HEPES, pH 7.2, 100 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM EDTA) containing a protease inhibitor mixture (Complete; Roche Diagnostics, Indianapolis, IN) by 5 sets of 30 strokes with a Dounce homogenizer (Wheaton Science Products, Millville, NJ). The homogenate was centrifuged at 2,500 rpm for 10 min at 4°C in a microcentrifuge to remove nuclei and unbroken cells. The postnuclear supernatant was centrifuged at 65,000 rpm for 80 min at 4°C in a Beckman TLA 100.2 rotor. The supernatant was used as the cytosol fraction. The pellet was then rinsed once with homogenization buffer, solubilized in homogenization buffer containing 1% Triton X-100, and centrifuged at 12,000 rpm for 20 min at 4°C in a microcentrifuge. The supernatant was used as the membrane fraction. The protein concentrations of the cytosol and membrane extracts were adjusted to 0.9 mg/ml with homogenization buffer. The extracts were incubated at 25°C for 30 min in the presence of 100 μM GDP or GTPγS, after which $MgCl_2$ was added to a final concentration of 7 mM. To reduce non-specific binding, the cytosol (350 μl) and membrane (170 μl) extracts were then preincubated overnight at 4°C with 20 μg of GST prebound to glutathione-Sepharose beads, and centrifuged at 2,500 rpm for 1 min in a microcentrifuge. The supernatants were then incubated for 3 h at 4°C with 10 μg of the fusion protein (GST–γ1-ear, GST–γ2-ear, or GST–αA-ear) prebound to glutathione-Sepharose beads and centrifuged at 2,500 rpm for 1 min. The pelleted beads were washed four times with homogenization buffer containing 0.5% Triton X-100, boiled in SDS-PAGE sample buffer, and centrifuged at 2,500 rpm for 1 min. Equivalent volumes of the supernatants were electrophoresed in a 7.5% SDS–polyacrylamide gel and electroblotted onto an Immobilon-P membrane (Millipore,

Bedford, MA). The blot was incubated sequentially with monoclonal mouse anti–Rabaptin-5 antibody (Clone 20; Transduction Laboratories, Lexington, KY) and with horseradish peroxidase–conjugated anti–mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and detected using a Renaissance Chemiluminescence reagent *Plus* (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions.

Coimmunoprecipitation Analysis—HeLa cells stably expressing HA-tagged Rabaptin-5 were established by transfection of the cells with pcDNA3-HA-Rbt5 using the FuGene6 transfection reagent (Roche Diagnostics) and subsequent selection in the presence of 800 μg/ml Geneticin (Life Technologies, Rockville, MD). One (HeLa/HA-Rbt5) of the clones with a moderate level of Rabaptin-5 expression was used for the following experiment. Non-transfected HeLa cells or HeLa/HA-Rbt5 cells grown to approximately 80% confluence in three 100-mm dishes were washed twice with ice-cold PBS and scraped into 0.7 ml of immunoprecipitation buffer (100 mM HEPES, pH 7.2, 1 mM $MgCl_2$, 50 mM NaF) containing a protease inhibitor mixture (Complete EDTA-free; Roche Diagnostics). The cells were then homogenized by twenty passages through a 22-gauge needle, and centrifuged at 2,500 rpm for 10 min at 4°C to remove nuclei and unbroken cells. The protein concentration of the postnuclear supernatant was adjusted to 2.0 mg/ml with immunoprecipitation buffer. The postnuclear supernatant (430 μl) was then incubated with 20 μl of anti–HA-conjugated agarose (HA-Probe; Santa Cruz Biotechnology, Santa Cruz, CA) or 0.5 μg of monoclonal mouse anti–Rabaptin-5 antibody (Clone 20) at 4°C for 18 h. The postnuclear supernatant incubated with anti–Rabaptin-5 was then incubated with Protein G–Sepharose 4FF (Amersham Pharmacia Biotech) at 4°C for 3 h. The incubation mixture was centrifuged at 2,500 rpm for 10 min at 4°C in a microcentrifuge. The pellet was washed four times with ice-cold wash buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% NP-40), boiled in SDS-PAGE sample buffer, electrophoresed in a 7.5% SDS–polyacrylamide gel, and electroblotted onto an Immobilon-P membrane. The blot was incubated sequentially with monoclonal mouse anti–γ1-adaptin antibody (100/3; Sigma Chemical, St. Louis, OH) and with horseradish peroxidase–conjugated anti–mouse IgG, and detected using a Renaissance Chemiluminescence reagent *Plus* according to the manufacturer's instructions.

Indirect Immunofluorescence Microscopy—HeLa cells or normal rat kidney (NRK) cells grown in the wells of an 8-well Lab-Tek II chamber slide (Nunc A/S, Roskilde, Denmark) were fixed and permeabilized as described by Neuhaus *et al.* (*45*). Briefly, to cells washed twice with ice-cold PBS, cold methanol at –80°C was added, and the temperature was raised to –20°C in 30 min. When indicated, the cells were incubated with 5 μg/ml BFA for 1 min prior to fixation. The fixed and permeabilized cells were washed twice with ice-cold PBS, and incubated with PBS at room temperature for 20 min, then with PBS containing 0.1% gelatin at room temperature for 1 h. The cells were then incubated sequentially with a combination of polyclonal goat anti–Rabaptin-5 antibody (Santa Cruz Biotechnology) and monoclonal mouse γ1-adaptin antibody [100/3 for HeLa cells or clone 88 (Transduction Laboratories) for NRK cells], and with a combination of Cy3-conjugated anti-goat and FITC-conjugated anti–mouse IgGs (Jackson ImmunoResearch Laboratories). To compare the distribution of γ1-adaptin with that of internalized transferrin, HeLa cells were rinsed briefly with serum-free medium and incubated with Alexa594-conjugated transferrin (a kind gift from Dr. Satoshi Waguri, Osaka University, Osaka) at 4°C for 30 min at a concentration of 5 μg/ml. Internalization was initiated by replacing the medium with fresh medium prewarmed at 37°C. At the end of internalization, the cells were rinsed briefly in ice-cold PBS, fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, and permeabilized with 50 μg/ml digitonin in PBS for 5 min at 4°C. The cells were then incubated sequentially with monoclonal mouse anti–γ1-adaptin antibody (100/3) and with Alexa488-conjugated anti–mouse IgG (Molecular Probes, Eugene, OR). To compare the localization of γ1-adaptin with that of either Rab4(Q67L) or Rab5(Q79L), HeLa cells were transfected with pcDNA3-HA-Rab4(Q67L) or pcDNA3-HA-Rab5(Q79L) using the FuGene6 reagent, incubated for 18 h in the case of the Rab4(Q67L)-transfected cells or 40 h in the case of the Rab5(Q79L)-transfected cells, fixed, and permeabilized with cold methanol. The cells were then incubated sequentially with a combination of monoclonal rat anti-HA antibody (3F10, Roche Diagnostics) and monoclonal mouse anti–γ1-adaptin antibody (100/3), and with a combination of Alexa488-conjugated anti–rat IgG (Molecular Probes) and Cy3-conjugated anti–mouse IgG (Jackson ImmunoResearch Laboratories). The stained cells were observed with a confocal laser-scanning microscope (TCS-NT; Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany).

RESULTS

Yeast Two-Hybrid Analysis of the Interaction between γ-Adaptin and Rabaptin-5—In order to identify proteins that interact with γ (γ1)-adaptin, reporter yeast cells were first transformed with a plasmid coding for a fusion protein between the GAL4 DNA-binding domain and full-length γ1-adaptin (pGBT-γ1; *14*). The cells were subsequently transformed with a plasmid mouse brain cDNA library coding for proteins as COOH-terminal fusions with the transcription activation domain of GAL4. Screening of approximately 1×10^6 transformants yielded several positive clones. One of the positive clones was found to contain a cDNA insert covering a polypeptide region (amino acid residue 276 to the COOH-terminus, residue 862) of mouse Rabaptin-5. As shown in Fig. 1B, retransformation of the positive clone [named pGAD-Rbt5(276-862)] revealed Rbt5(276-862) to be able to interact with γ1-adaptin (pGBT-γ1) but not with the empty pGBT9 vector. As expected, γ1-adaptin was also able to interact with full-length Rabaptin-5 [Rbt5-(full)]. We also constructed a pGBT9-based vector for Rbt5-(full) and a pGAD-based vector for γ1-adaptin, cotransformed them into reporter cells, and found a positive interaction (data not shown). These results indicate that γ1-adaptin and Rabaptin-5 are able to interact specifically with each other.

Rabaptin-5 has four potential coiled-coil regions (*31, 38*), two in the NH_2-terminal region (CC1-1 and CC1-2) and the other two in the COOH-terminal region (CC2-1 and CC2-2). The originally identified clone, Rbt5(276-862), lacks the NH_2-terminal coiled-coil regions (see Fig. 1A). In order to

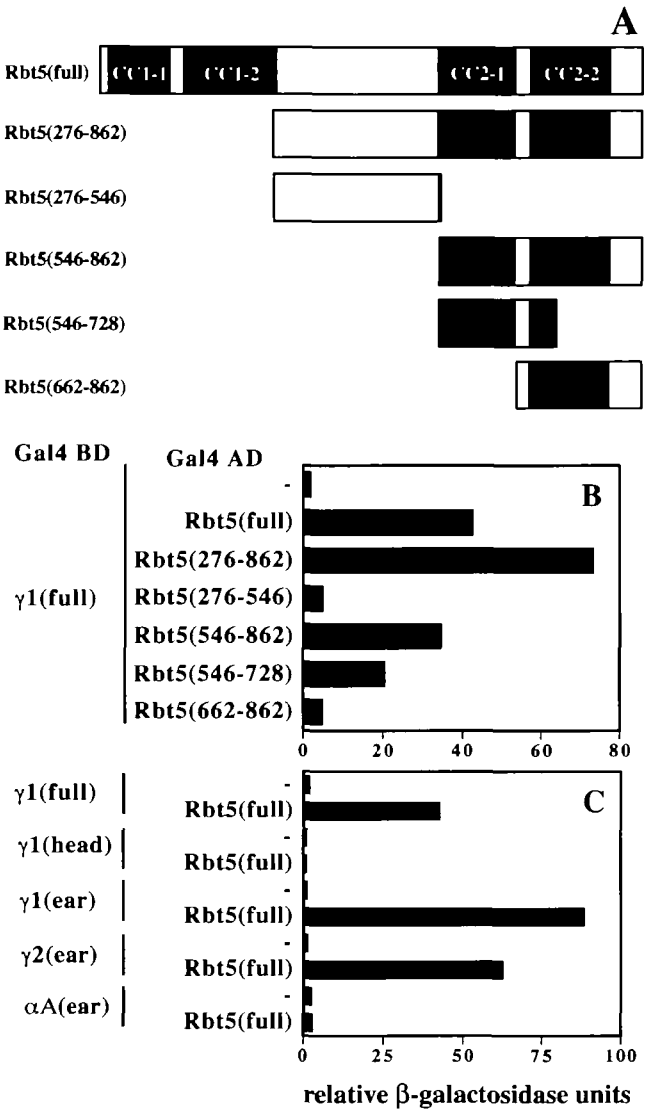

Fig. 1. **Interaction between γ-adaptin and Rabaptin-5 revealed by the yeast two-hybrid system.** A, schematic representation of the structures of mouse Rabaptin-5 (Rbt5) and its deletion mutants. B, interaction between full-length γ1-adaptin and Rabaptin-5 or its deletion mutants. Reporter yeast cells cotransformed with the pGBT9-based (GAL4 BD) full-length γ1-adaptin vector and either of the pGAD10-based (GAL4 AD) Rabaptin-5 vectors as indicated were subjected to liquid β-galactosidase assay as described under "MATERIALS AND METHODS." C, interaction between adaptin fragments and full-length Rabaptin-5. Reporter yeast cells cotransformed with either of the pGBT9-based adaptin vectors as indicated and the pGAD10-based full-length Rabaptin-5 vector or empty vector were assayed as described above.

determine the region responsible for the interaction with γ1-adaptin, we therefore divided Rbt5(276-862) into two regions, one [Rbt5(276-546)] covering the central featureless region and the other [Rbt5(546-862)] covering the COOH-terminal coiled-coil regions (Fig. 1A). As shown in Fig. 1B, Rbt5(546-862), but not Rbt5(276-546), interacted with γ1-adaptin. We then constructed Rbt5(546-728) and Rbt5(662-862) (Fig. 1A) and found that the former but not the latter interacted with γ1-adaptin. These results indicate that at least the CC2-1 region of Rabaptin-5 is responsible for its interaction with γ1-adaptin, although another region may also contribute to the interaction because the β-galactosidase activity detected using Rbt5(546-728) was lower than that using the originally identified clone, Rbt5-(276-862).

We then set out to determine which region of γ1-adaptin is responsible for its interaction with Rabaptin-5. The γ1-adaptin polypeptide is structurally divided into two domains, the NH_2-terminal head or trunk domain and the COOH-terminal ear or appendage domain, which are connected by a proline-rich hinge region (*4–6*). We therefore constructed pGBT9-based vectors for the head and ear domains and cotransformed each of them with pGAD-Rbt5(full) into reporter yeast cells. As shown in Fig. 1C, the ear domain of γ1-adaptin showed a strong interaction with Rabaptin-5. In contrast, the interaction between the head domain and Rabaptin-5 was below the detection level. Because the ear domains of γ1- and γ2-adaptins are highly conserved (*14*), we then examined whether the ear domain of γ2-adaptin is also able to interact with Rabaptin-5. As expected, the ear domain of γ2-adaptin also showed a significant interaction with Rabaptin-5 in the two-hybrid assay (Fig. 1C). In contrast, the ear domain of αA-adaptin, which does not show significant homology with either γ1- or γ2-adaptin, showed no significant interaction.

In Vitro and In Vivo Interactions between γ1-Adaptin and Rabaptin-5—In order to examine whether the interaction between γ1-adaptin and Rabaptin-5 can be detected biochemically, we prepared fusion proteins between glutathione *S*-transferase (GST) and the ear domains of γ1-, γ2-, and αA-adaptins, and immobilized them on glutathione-Sepharose beads. In this experiment, a GST fusion protein with αA-adaptin covering a portion of the hinge region and the entire ear domain was used in place of the ear domain alone for the reason described in "MATERIALS AND METHODS." Subsequently, the beads were incubated with cytosol and membrane extracts from HeLa cells. GDP or GTPγS was included in the incubation mixture because we suspected that a Rab GTPase might regulate the interaction. After washing the beads, we analyzed the bound materials by SDS-PAGE and Western blotting with anti–Rabaptin-5 antibody. As shown in Fig. 2, a significant fraction of cytosolic and membrane-associated Rabaptin-5 bound to the GST fusion proteins with the ear domains of γ1- and γ2-adaptins (lanes 2, 5, 9, and 12, and lanes 3, 6, 10, and 13, respectively). In contrast, Rabaptin-5 did not bind to the GST fusion protein with the ear domain of αA-adaptin (lanes 1, 4, 8, and 11) or to GST alone (data not shown), suggesting that Rabaptin-5 binding is specific to the ear domains of γ1- and γ2-adaptins. It is of note that no significant difference was observed in the amount of Rabaptin-5 bound to the GST-γ-adaptin-ear fusion under either the GDP- and GTPγS-containing conditions. Furthermore, Rab4 or Rab5 was not pulled down with the ear domain (data not shown).

In an attempt to investigate whether Rabaptin-5 is able to interact with γ1-adaptin *in vivo*, we subjected the post-nuclear supernatant from HeLa cells to immunoprecipitation with anti–Rabaptin-5 or anti–γ1-adaptin antibody, and then performed SDS-PAGE and Western blotting with the reversal antibody to determine whether these proteins co-immunoprecipitated. However, only a faint band for γ1-adaptin and no band for Rabaptin-5 were detected (data

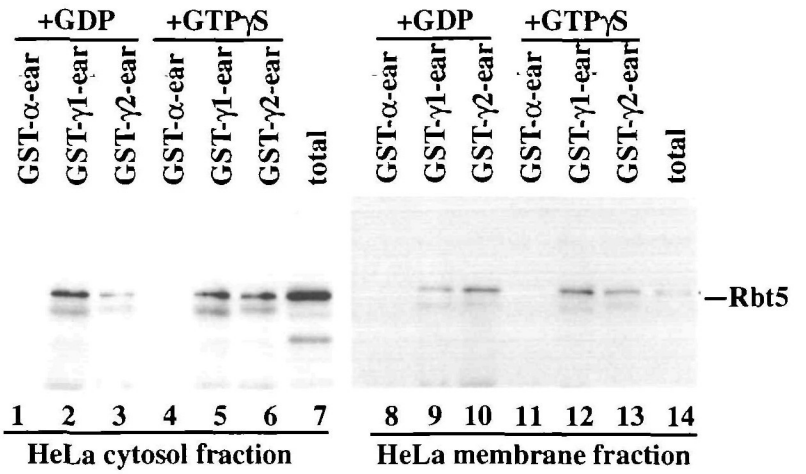

Fig. 2. **Pull down assay.** The ear domain of γ1-, γ2, or αA-adaptin fused to GST as indicated was immobilized on glutathione-Sepharose beads, incubated with cytosol or the membrane extract of HeLa cells in the presence of GDP or GTPγS, and subjected to Western blotting with anti–Rabaptin-5 antibody as described under "MATERIALS AND METHODS" to study their *in vitro* binding to Rabaptin-5.

not shown), probably because the expression levels of the endogenous proteins were too low to be detected using the antibodies. We, therefore, established a HeLa cell line stably expressing hemagglutinin (HA)-tagged Rabaptin-5 (HeLa/HA-Rbt5), prepared the postnuclear supernatant of this cell line, and subjected it to immunoprecipitation with anti-HA or anti–Rabaptin-5 antibody. As shown in Fig. 3, subsequent SDS-PAGE and Western blotting with anti–γ1-adaptin antibody revealed γ1-adaptin to coimmunoprecipitate with both the anti-HA and anti–Rabaptin-5 antibodies (lanes 3 and 5, respectively). When the postnuclear supernatant from non-transfected HeLa cells was subjected to immunoprecipitation with anti-HA and anti–Rabaptin-5 antibodies, no band (lane 2) and a faint band (lane 4) for γ1-adaptin, respectively, were detected. Essentially the same results were obtained using the postnuclear supernatant from HeLa/HA-Rbt5 cells transiently overexpressing Rab4(Q67L) or Rab5(Q79L) (data not shown). These results indicate that the interaction between γ1-adaptin and Rabaptin-5 is specific *in vivo*.

Rabaptin-5 Colocalizes with γ1-Adaptin in the Perinuclear Region—Recently, using an improved fixation method (*45*) it has been shown that endogenous Rabaptin-5 is localized not only to punctate, but also to perinuclear endosome-like structures (*46*). On the other hand, γ1-adaptin has been shown to localize to endosome-like structures as well as the TGN (*8–11*). We therefore examined whether γ1-adaptin and Rabaptin-5 colocalize in the cell by double staining for endogenous proteins. In HeLa cells (Fig. 4, A-A″) and NRK cells (B-B″) fixed and permeabilized by the method of Neuhaus *et al.* (*45*; see "MATERIALS AND METHODS"), the staining for Rabaptin-5 was found to be in the perinuclear region, where the staining was overlapped significantly with that for γ1-adaptin, although the Rabaptin-5–staining appeared to be rather broader than the γ1-adaptin staining, and cytoplasmic staining for Rabaptin-5 was also apparent. This observation suggests the possibility that the γ1-adaptin–Rabaptin-5 interaction takes place in cells.

We then treated the cells with brefeldin A (BFA), a fungal metabolite, and observed the distribution of γ1-adaptin and Rabaptin-5. BFA is known to inhibit guanine nucleotide exchange factors for ADP-ribosylation factor (ARF), and thereby inhibit membrane binding of ARF (for reviews,

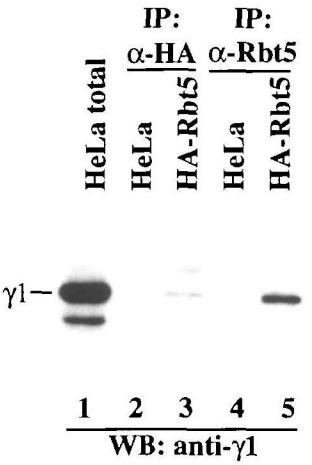

Fig. 3. **Coimmunoprecipitation analysis.** Lysates from HeLa cells or HeLa/HA-Rbt5 cells were immunoprecipitated (IP) with anti-HA (α-HA) or anti–Rabaptin-5 (α-Rbt5) antibody and the immunoprecipitates were subjected to Western blotting (WB) with anti–γ1-adaptin as described under "MATERIALS AND METHODS" to study the *in vivo* interaction between γ1-adaptin and Rabaptin-5. The position of the co-immunoprecipitated γ1-adaptin is indicated. Although the origin of the faint band migrating more slowly than the γ1-adaptin band (lanes 3 and 5) is unclear, the band may represent HA-Rabaptin-5 concentrated in the immunoprecipitates that cross-reacted weakly with the γ1-adaptin antibody, because the position of the band corresponds to that of the Rabaptin-5 and the band was reproducibly observed in the immunoprecipitates of the lysates of cells expressing HA-Rabaptin-5.

see Refs. *47* and *48*). Because the association of the AP-1 complex with membranes is dependent on ARF, treatment of cells with BFA causes the dissociation of AP-1 from membranes (*49–51*). Although BFA also causes the redistribution of transmembrane Golgi proteins, the redistribution requires much longer BFA treatment (more than 5 min) than that required for the dissociation of ARF and coat proteins from membranes (within 30 s) (*51*). Therefore, we speculated that a relatively short-term treatment of cells with BFA would give rise to redistribution of not only γ1-adaptin but also Rabaptin-5 into the cytoplasm if the membrane association of Rabaptin-5 was mediated by its binding to γ1-adaptin. As shown in Fig. 4, C-C″, this was the

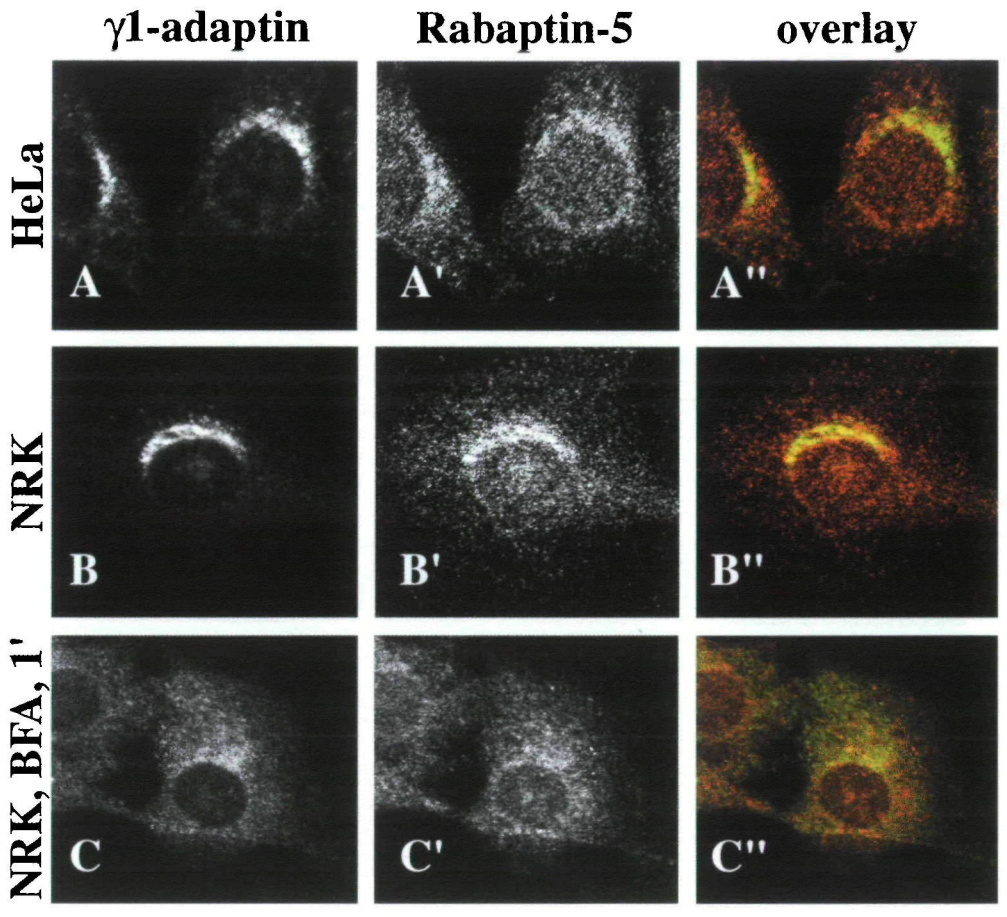

Fig. 4. **Colocalization of endogenous γ1-adaptin and Rabaptin-5.** HeLa cells (A-A″) or NRK cells (B-B″ and C-C″) were directly fixed (A-A″ and B-B″) or incubated with 5 μg/ml BFA for 1 min prior to fixation (C-C″), and subjected to double-staining for γ1-adaptin (A, B, and C) and Rabaptin-5 (A′, B′, and C′) as described under "MATERIALS AND METHODS." Overlays are shown in A″, B″, and C″ (green for γ1-adaptin and red for Rabaptin-5).

case; when NRK cells were treated with 5 μg/ml BFA for 1 min, both γ1-adaptin and Rabaptin-5 redistributed from the perinuclear region to the cytoplasm. In contrast, such treatment did not affect the distribution of a Golgi-resident protein, mannosidase II, nor that of a peripheral early endosomal protein, EEA1 (data not shown), indicating that the effect of short-term BFA treatment is specific for γ1-adaptin and Rabaptin-5. Thus, the membrane association of γ1-adaptin and Rabaptin-5 appears to be a coupled event. Taken together with the above data on the interaction between γ1-adaptin and Rabaptin-5, it seems likely that at least some fraction of Rabaptin-5 molecules associates with membranes in a γ1-adaptin–dependent manner, although the formal possibility that the Rabaptin-5 association is directly regulated by ARF cannot be rigorously excluded.

Localization of γ1-Adaptin on Endosomal Compartments—We then set out to examine whether γ1-adaptin is localized on endosomes in our system. To this end, we took two approaches. First, we compared the distribution of γ1-adaptin with that of internalized transferrin, because it is known to reach early endosomes at an earlier time of internalization and recycling compartments at later times, then return to the cell surface (for review, see Ref. *52*). As shown in Fig. 5, after 2 min of internalization, Alexa594-conjugated transferrin was found in punctate endosomal structures distributed throughout the cytoplasm. At this time point, there was little overlap between transferrin and γ1-adaptin (A-A″). After 10 min of internalization, however, Alexa594-transferrin began to accumulate not only in peripheral punctate structures, but also in perinuclear structures (data not shown). After 20 min of internalization, the transferrin labeling was significantly superimposed on the perinuclear γ1-adaptin–containing structures (B-B″). Then, we exploited the fact that the overexpression of Rab5(Q79L) or Rab4(Q67L) leads to an exaggeration of structures containing Rabaptin-5 and its variant Rabaptin-4 (*31, 39*), although Rab5 and Rab4 are localized to distinct but partially overlapping endosomal compartments, peripheral early endosomes and perinuclear recycling endosomes, respectively (*39, 40, 42*). In HeLa cells overexpressing HA-Rab4(Q67L), the small GTPase and γ1-adaptin colocalized significantly, although partially, on perinuclear structures (C-C″). In contrast, the localization of γ1-adaptin on exaggerated early endosomal structures containing overexpressed Rab5(Q79L) was barely observable (D-D″). These observations indicate that at least a population of γ1-adaptin molecules localizes on endocytic compartments, probably recycling endosomes, as well as on the TGN.

DISCUSSION

The present study provides the first evidence for a direct interaction between γ-adaptins and Rabaptin-5. In yeast two-hybrid screening, we found that γ1-adaptin is able to interact with Rabaptin-5. Further two-hybrid analysis (Fig. 1) and pull down assay (Fig. 2) showed that the ear domains of both γ1- and γ2-adaptins interact with the CC2-

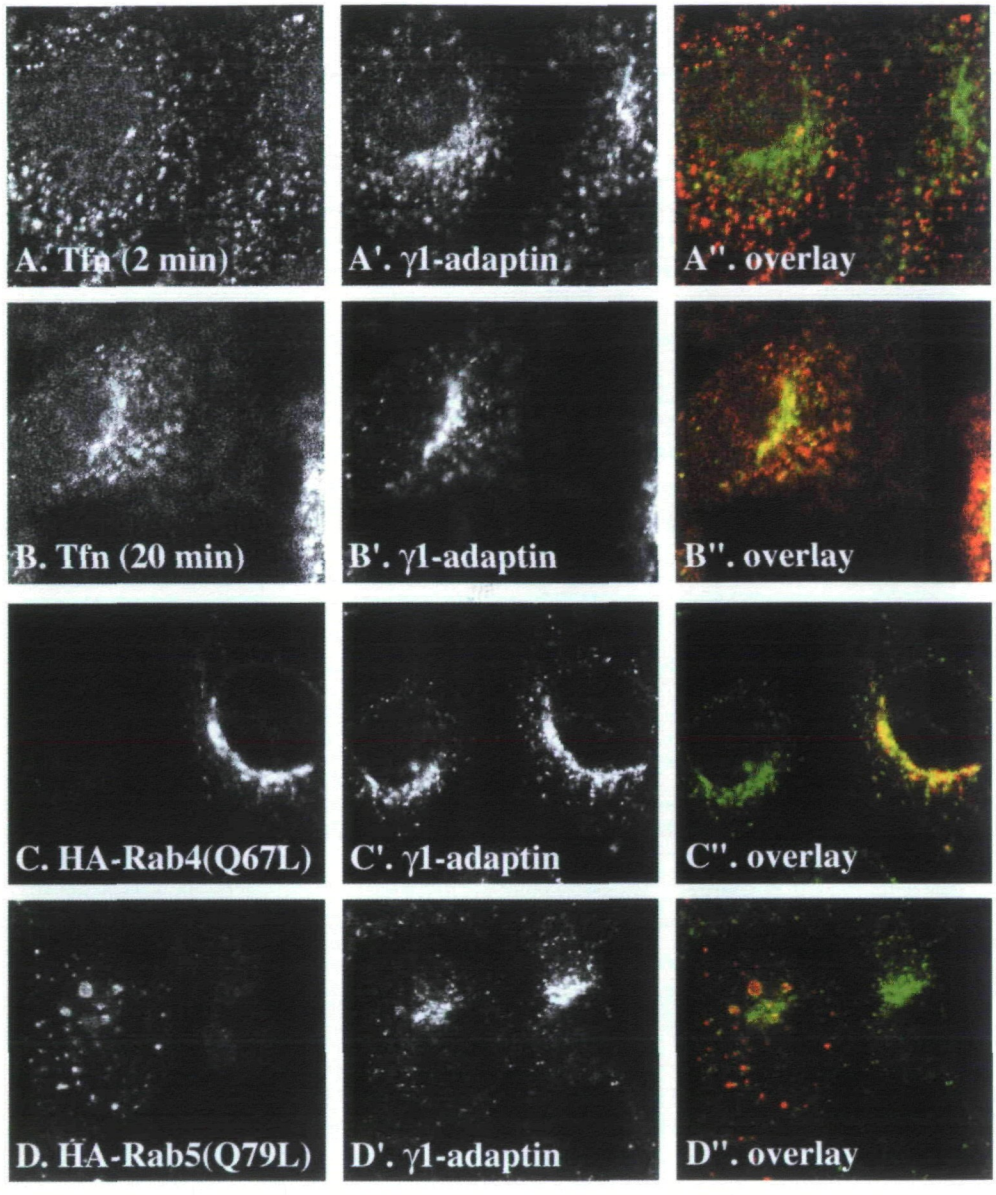

Fig. 5. **Localization of γ1-adaptin on endosomes.** A and B, HeLa cells preloaded with Alexa594-conjugated transferrin (Tfn; A and B) were incubated at 37°C for 2 (A-A") or 20 (B-B") min, fixed and processed for immunofluorescence to visualize γ1-adaptin (A' and B') as described under "MATERIALS AND METHODS." C and D, HeLa cells transfected with pcDNA3-HA-Rab4(Q67L) (C-C") or pcDNA3-HA-Rab5(Q79L) (D-D") were subjected to double-staining with anti-HA (C and D) and anti-γ1-adaptin (C' and D') antibodies as described under "MATERIALS AND METHODS." Overlays are shown in A"-D" (green for γ1-adaptin and red for transferrin or HA-Rab).

1 region of Rabaptin-5. The significance of the interaction between γ1-adaptin and Rabaptin-5 in cells is corroborated by the results that Rabaptin-5 and γ1-adaptin can be coimmunoprecipitated from cell lysates (Fig. 3) and that immunofluorescence analysis revealed their significant colocalization on perinuclear structures, probably on recycling compartments (Fig. 4). However, we were unable to examine the physiological relevance of the interaction between γ2-adaptin and Rabaptin-5 due to the limitations of the antibodies to γ2-adaptin we raised, which do not work well in immunoprecipitation nor detect endogenous protein in immunofluorescence analysis (*14*).

On the basis of the data presented here, at least two explanations are possible for the role of Rabaptin-5 in the function of clathrin-coated vesicles containing the AP-1 complex. One is that clathrin-coated vesicles that are derived from the TGN become associated with Rabaptin-5 either in the cytosol or on membranes, which in turn leads to docking and fusion of the vesicles with endosomes by forming oligomeric complexes with EEA1, Rabex-5, and NSF (*35*; for review, see Ref. *30*). This possibility is indirectly supported by the recent report of Rubino *et al.* showing that docking and fusion between plasma membrane-derived clathrin-coated vesicles and early endosomes requires the prior recruitment of Rabaptin-5 onto both membranes and that of EEA1 onto endosomal membranes (*53*). It is therefore tempting to speculate that TGN-derived clathrin-coated vesicles associated with Rabaptin-5 are also competent to dock and fuse with endosomes. Recent data showing that EEA1 interacts directly not only with syntaxin 13 (*35, 37*), a SNARE implicated in plasma membrane–early endosome trafficking (*35, 53, 54*), but also with syntaxin 6 (*36, 37*), a SNARE associated with TGN-derived clathrin-coated vesicles that is implicated in TGN–endosome trafficking (*55*), is in line with this speculation.

The other possible explanation is that Rabaptin-5 is associated with endosome-derived clathrin-coated vesicles. Although AP-1 is mainly associated with the TGN, it has

been shown also to be associated with endosomes (*8–11*). The present study shows that at least a population of AP-1 localizes on Rab4-containing recycling compartments (Fig. 5). Furthermore, a recent study using cells derived from mice lacking the μ1 subunit of the AP-1 complex has unexpectedly highlighted the crucial role of AP-1 in retrograde endosomes to TGN trafficking, rather than in anterograde TGN to endosome trafficking, of mannose 6-phosphate receptors (*56*). Taken together, it is possible that clathrin-coated vesicles derived from endosomes may dock and fuse with the TGN or other compartment(s) through their association with Rabaptin-5.

In either case, it is an interesting issue whether the interaction between γ1-adaptin and Rabaptin-5 is regulated by Rab GTPases, because Rabaptin-5 and its variant Rabaptin-4 interact with GTP-bound Rab5 and Rab4 (*31, 38, 39*), and because the recruitment of Rabaptin-5 onto early endosomes and plasma membrane–derived clathrin-coated vesicles is dependent on Rab5 (*53*). Furthermore, McLauchan *et al.* have shown that a complex of Rab5 and a guanine-nucleotide dissociation inhibitor is required for plasma membrane clathrin-coated pits to sequester ligand in an adaptor-dependent manner and suggested that the recruitment of components essential for vesicle targeting and fusion is coupled to the formation of functional transport vesicles (*57*). Our immunofluorescence analysis indicates that at least a population of γ1-adaptin molecules is localized to recycling compartments containing Rab4-(Q67L), but not to early endosomes containing Rab5(Q79L) (Fig. 5). This observation is in line with evidence for the association of AP-1 with GLUT4-containing vesicles (*58*), with which Rab4 is also known to associate (*59*). It is therefore possible that Rab4 may regulate the recruitment of Rabaptin-5/Rabaptin-4 onto nascent clathrin/AP-1–coated vesicles. However, our biochemical analyses failed to show a direct involvement of Rab GTPase in the regulation of the γ1-adaptin–Rabaptin-5 interaction. First, the GST fusion protein with the γ1-adaptin-ear domain pulled down Rabaptin-5 to the same extent under both GDP- and GTPγS-containing conditions (Fig. 2). Second, the GST-γ1-adaptin-ear fusion protein did not pull down Rab4 or Rab5 together with Rabaptin-5 (data not shown). Finally, our preliminary experiment revealed that the overexpression of Rab4-(Q67L) or Rab5(Q79L) does not affect the coimmunoprecipitation efficiency of Rabaptin-5 and γ1-adaptin (Shiba, Y. and Nakayama, K., unpublished results). However, these data do not necessarily exclude the possibility that Rab GTPase may play a role in the interaction between γ1-adaptin and Rabaptin-5, because Rab GTPases do not remain stably bound to effectors (*60*).

Zerial and colleagues have proposed, on the basis of their data showing that EEA1 alone can support docking and fusion between endosomes (*33*), and that a prior complex formation between Rabaptin-5 and Rabex-5 is essential for the activation of Rab5 and endosome fusion (*61*), that the Rabaptin-5/Rabex-5 complex may ensure the activation of Rab5 on endosomes and the activated Rab5 may in turn regulate EEA1 recruitment and activity. Furthermore, Cao and Barlowe have recently shown, by an *in vitro* docking and fusion assay using combinations of vesicles and target membranes derived from wild-type yeast cells and temperature-sensitive mutants, that Ypt1p, a yeast ortholog of Rab1, is required on target membranes and not on vesicles for membrane docking and fusion (*62*, reviewed in Ref. *63*). Taken together, it is possible that the Rabaptin-5/Rabex-5 complex associated with clathrin-coated vesicles may regulate Rab4 on target membranes rather than Rab4 regulating Rabaptin-5 association onto vesicles. However, we were unable to show evidence for an association with γ1-adaptin of Rabex-5 together with Rabaptin-5 due to the unavailability of an anti-Rabex-5 antibody; it is currently unknown whether Rabex-5 is also active on Rab4 due to the high, intrinsic guanine-nucleotide exchange rate of Rab4 *in vitro* (*64*). Further experiments will be required to address this issue.

Recently, a family of proteins, designated GGA1, GGA2, and GGA3, which have in common a COOH-terminal domain homologous to the ear domain of γ-adaptin, has been identified and shown to localize on the *trans*-Golgi and/or TGN (*65–69*, reviewed in Refs. *70* and *71*). Furthermore, the ear-like domains of the GGA proteins have been shown to interact with γ-synergin (*67, 69*), which is known to interact with the ear domain of γ-adaptin (*25*). It is therefore an interesting issue whether the GGA ear-like domains are also able to interact with Rabaptin-5. Our preliminary two-hybrid analysis indicates that such an interaction can occur (Y. Shiba, H. Takatsu, and K. Nakayama, unpublished results), suggesting that the ear (ear-like) domains of γ-adaptins and GGAs, like that of α-adaptin (*19*), may regulate membrane trafficking by interacting with various regulatory molecules. It is a future issue to be addressed whether γ-adaptins and GGAs cooperatively or independently regulate membrane trafficking events.

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